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Proteomic Protease Substrate Profiling of tPA Treatment in Acute Ischemic Stroke Patients: A Step Toward Individualizing Thrombolytic Therapy at the Bedside

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Abstract

Tissue plasminogen activator (tPA) is the only FDA-approved medical therapy for acute ischemic stroke. But as a serine peptidase, intravenous tPA can affect the expression of other proteases that may be implicated in blood–brain barrier breakdown. Such parallel cascades of cell signaling may be involved in intracranial hemorrhage, the major side effect of tPA. Here, we describe an initial attempt in proteomic substrate profiling, i.e., degradomics in human plasma within the context of acute stroke. Plasma from acute stroke patients were analyzed pre- and post-intravenous tPA using tandem mass spectrometry and protein array profiling to identify substrates and proteases of interest. In non-tPA-treated stroke plasma, degradomic patterns indicated a rapid induction of protease activity within 3 h of stroke onset that mostly stabilized by 24 h. But in tPA-treated patients, pre- and post-tPA samples from the same patient demonstrated distinct degradomic patterns that persisted even up to 3–5 days after stroke onset. Matching control patients without strokes had little change in degradomic profiles over time. Our findings demonstrate that tPA treatment changes the plasma degradomic profiles in acute stroke patients. These composite proteolytic profiles may provide a glimpse of the pleiotropic effects of tPA on cellular signaling cascades at the bedside. This study supports the feasibility of performing pharmaco-proteomics at the bedside, which may ultimately allow us to dissect mechanisms of thrombolysis-related therapeutic efficacy in stroke.

Keywords

Proteomics; Mass spectrometry; Blood–brain barrier; Matrix metalloproteinase; Neuroprotection; Translational; Biomarker

Introduction

Thrombolysis with intravenous (IV) tissue plasminogen activator (tPA) is currently the only FDA-approved medical therapy for acute ischemic stroke. Although efficacious in increasing the proportion of patients with better neurologic outcome by 30–35% at 12 months, tPA is only given to less than 5% of stroke patients, due to its dreaded side effect of tenfold increased risk of intracranial hemorrhage (ICH), with more than 50% mortality rate for patients with major hemorrhage [1]. Although tPA-induced ICH carries a high mortality rate, the benefit of early reperfusion by tPA still outweighs the risks [2–7]. Recent clinical trials and further meta-analysis demonstrate a benefit even at 4.5 h post initial stroke symptom onset [8–14]. Clinical characteristics, imaging criteria, and biomarkers have been studied to predict tPA-related outcome [15–22]. However, the optimum criteria to select individual patients who may receive the maximum therapeutic efficacy and the least side effects, without the rigid time-window boundary, remain uncertain. In order to maximize the benefit of thrombolysis by further widening the treatment window and also to evaluate new thrombolytic agents as they become available, it is imperative to better understand the mechanisms by which thrombolysis affects clinical outcomes [23].

tPA, a serine peptidase, has pleiotropic effects regulating the expression of matrix metalloproteinases (MMPs) and other proteases involved in cell–cell signaling in the brain. For example, tPA may increase the expression of matrix metalloproteinase 9 (MMP-9), which is implicated in the breakdown of the blood–brain barrier (BBB) leading to ICH [16, 17, 24–33]. While previous studies of bedside biomarkers have been limited to known individual factors such as MMP-9, the MMP–tPA axis has a variety of pleiotrophic interactions [23]. New proteomic methods and technology may help to identify both known and unknown factors at the same time, perhaps improving our understanding of thrombolytic

therapy with respect to protease and cell signaling interaction, a concept particularly enticing for disease entities such as stroke, which most likely involve multiple gene interactions [34]. One novel method, in particular, is the study of protease "degradomics" or substrate profiling: the investigation of, and techniques for characterizing, the "substrate repertoire" of proteases of interest [35, 36]. This method is particularly attractive for the study of multiple protease interactions, and it allows us to understand the biologic role of proteases both from an individual and global perspective [37].

We report here a first attempt of proteomic substrate profiling (degradomics) in stroke patient plasma, to understand better the effect of tPA on cell–cell signaling in the brain in the context of acute stroke pathophysiology in order to ultimately elucidate the mechanisms of the therapeutic efficacy and side effects of thrombolysis.

Methods

Clinical Sample Collection

A total of 39 plasma samples were obtained from tPA-treated acute stroke patients, pre-tPA and post-tPA administration and over time, from stroke patients who did not receive tPA and from non-stroke patients with similar risk factors. Subjects were matched with respect to age, gender, and stroke severity (see Table 1). Patient blood was drawn at three time points —post-stroke in the hyperacute (T1 <3 h), acute (T2 <24 h), and subacute stages (T3: 3–5 days). Samples were obtained from venous puncture and immediately processed to deep freeze at −80°C within 30 min of collection to ensure minimal protein degradation.

Proteomic Plasma Measurements

Samples were carefully aliquoted at time of collection to prevent repeated freeze-thaw cycles. All samples were processed at the same time by investigators blind to the clinical data to avoid bias and batch variations. Standard operating procedures were strictly observed for all samples. Patient plasma was thawed at room temperature and processed for mass spectrometry (MS) analysis [38]. Samples were reduced and alkylated by diluting with 8 M guanidine–HCl 100 mM ammonium bicarbonate 5% *n*-propanol 10 mM DTT. All samples were heated to 95°C for 30 min, 37°C for 1 h, and cooled to RT. Iodoacetic acid (500 mM) in 1 M ammonium bicarbonate was added and samples were allowed to react in the dark for 1 h. Subsequently, each sample received 1.6 M DTT and was vortexed and centrifuged at 14,000 RCF for 10 min. Each sample was injected using an autosampler on an AKTA Explorer onto a Superdex 200 10/300 column running 250 μL/min 100 mM Tris–HCl, 2.5 mM CaCl2, and 10% *n*-propanol. Fractions collected were separated by MW filters according to size and run on a Hybrid Linear Ion Trap Fourier Transformation Mass Spectrometer (LTQ-FT, Thermo-Finnigan) set at 400 K resolution, with MS2 and MS3 data acquired. We postulate that the later fractions, which contain smaller MW fragments, will contain in part the protease degradome of interest.

To quantitatively measure low-abundance proteases of interest in the plasma of acute stroke patients (tPA-treated, untreated) and healthy controls, individual plasma samples were analyzed using Searchlight Protein Microarrrays (Pierce). These validated protein micro-arrays confirm the presence and quantify the expression of low-abundance proteases of interest such as MMPs and their inhibitors (MMP1, 2, 3, 7, 8, 9, 10, and 13 and TIMP1 and TIMP2).

Results

Following size exclusion chromatography (SEC), individual plasma samples from various patients showed distinct patterns corresponding to the composition of peptides of differing

sizes. Elution profiles of control, untreated stroke patient, and tPA-treated stroke patients are shown in Fig. 1. Control plasma obtained from healthy adults had stable and unchanging degradomic patterns over time (Fig. 1a). In acute stroke patients who did not receive tPA or other medical intervention, there is a rapid change in the amount of protein expression within 3 h of onset (Fig. 1b). But this hyperacute response appeared to be transient and degradomic patterns stabilized by 24 h (Fig. 1b). In contrast, tPA-treated stroke patients demonstrated the most “active” degradomic patterns over time compared to those of untreated and control patients (Fig. 1c). Interestingly, from our chromatography examination of all 39 plasma samples, we found that all tPA-treated patients consistently demonstrated a distinctive pattern at the acute stage post-tPA (T2, <24 h post-stroke)—shown in circle on Fig. 1c. These unique changes captured from the size exclusion chromatography elution profiles may indicate increased amounts of smaller protein fragments found in patient plasma after stroke plus tPA treatment, with enhanced degradomic patterns persisting even up to 3–5 days later (Fig. 1c). This unique and interesting pattern appeared in the degradomic region of plasma in all post-tPA patients, where protease substrates related to tPA signaling cascades would most plausibly be expected to be found. Taking into account the half life of tPA, we hypothesized that these substrates may be most clinically relevant. Therefore, we specifically conducted proteomic analysis of the corresponding degradomic fractions in a representative tPA patient, the results of which are shown in Table 2.

An in-depth proteomic analysis of the individual degradomic fractions revealed a wide spectrum of proteins in the tPA-treated stroke patient plasma. Table 2 lists the names of individual protein fragments identified in these eluted fractions (fractions 7, 8, and 9) post-tPA. Many proteins, both peripheral from plasma and brain-specific, appeared to come from extracellular matrix, cell membrane, and secreted factor families important to cell–cell signaling and BBB breakdown and relevant to fibrinolytic pathways (eg., fibrinogen subunits, alpha2-macroglobulin, transthyretin, spectrin, utrophin (UTRN), and brain-specific angiogenesis inhibitor (BAI-1); see Table 2).

Because there is a strong link between tPA and MMPs, we explored the hypothesis that proteases from the MMP family may be involved in these detected degradomic patterns in our stroke patient plasma. Due to the low-abundance nature of MMPs in plasma, we were not able to detect reliable MMP signals during the high-throughput proteomic analysis of the larger eluted plasma fractions. However, targeted microarray testing confirmed that multiple MMPs were indeed present in plasma of both stroke patients and controls. Ranked levels of multiple MMPs and their endogenous inhibitors (graphed by circular plot to better visualize global changes—Fig. 2) demonstrated coordinated changes in control, tPA-treated, and untreated stroke patient plasma at hyperacute (<3 h), acute (<24 h), and subacute (3–5 days) stages. Healthy individuals had stable MMP levels over time, which were similar in men and women (Fig. 2a). Ranking was used to indicate relative amount of each protein for better visualization. Circular plots graphed according to rank levels of MMPs, and their inhibitors showed a general upregulation of several MMPs post-tPA (at hyperacute: T2—in red) in comparison to patients who did not receive tPA. (Fig. 2b, c). A network of MMPs, rather than individual MMPs, was elevated post-tPA (Fig. 2c).

Discussion

Experimental and clinical studies suggest that there is a mechanistic link between tPA therapy and MMP-9 after stroke [16, 17, 27–33]. In this study, a more general degradomic approach coupled with targeted protein arrays found differential protease substrate patterns, changing over time, in tPA-treated and untreated stroke patients. These findings suggest that beyond MMP-9 alone, tPA may trigger complex and dynamic responses in a wider range of proteases that can be detected at the bedside in patient plasma. Confirmation of low-

abundance proteases such as MMPs and their endogenous inhibitors such as tissue inhibitor of metalloproteinases (TIMPs) provides a window into the coordinated signaling responses after stroke that may eventually impact the balance between treatment efficacy and associated risks of thrombolysis-related complications such as ICH and edema.

Emerging data now suggest that thrombolysis-related reperfusion injury may be partly attributable to some of the potentially neurotoxic side effects of the thrombolytic agent itself in the signaling action within the neurovascular unit [15–17, 25, 26, 39]. Apart from its intended role in clot lysis, tPA —a serine peptidase itself—may also act as an extracellular signaling molecule in the brain. By augmenting MMP dysregulation after stroke, tPA may degrade extracellular matrix integrity and increase risks of neurovascular cell death, BBB leakage, edema, and hemorrhage in both animal models and clinical studies [16, 24–26, 33]. Imaging data offer new evidence demonstrating BBB disruption, visible as delayed gadolinium contrast leakage into the cerebral spinal fluid (CSF) space on MRI fluid-attenuated inversion recovery sequences, to be associated with hemorrhagic transformation, MMP-9, and worse clinical outcome [40, 41]. However, others have reported that the same processes can also actually be beneficial; minor thrombolysis-related HT (HI1–HI2) not only represented a marker of early successful recanalization but also led to a reduced infarct size and improved clinical outcome [15]. These findings suggest the complexity of the molecular interplay responsible for thrombolysis-related effects and the need to further study tPA's intravascular mechanism at the bedside utilizing a systems approach such as proteomics.

In the discovery proteomic profiling of tPA-treated samples, we found known thrombolysis pathway-related proteins, such as fibrinogen and alpha2 macroglobulin, to be degraded [42]. We were able to identify different components of fibrinogen subunits in the post-thrombolytic profiles (alpha and gamma subunits). Moreover, good markers of damaged BBB and ischemic brain injury (eg., transthyretin, spectrin) were found to be present post-tPA [43, 44]. In addition to known substrates related to the tPA–MMP axis, novel substrates potentially relevant to vessel integrity and brain plasticity were also identified. Brain-specific angiogenesis inhibitor (BAI-1), a signaling factor relevant for angiogenesis, and utrophin, a membrane-bound protein in the scaffolding of mammalian brain with potential role in post-synaptic membrane maintenance, are both potential candidates of interest (Table 2). These substrates will need further validation and quantification.

Hence, in order to improve thrombolysis therapy and minimize its detrimental effects, targeting a single mechanism may not be sufficient. Combinatorial approaches will be necessary, due to the complex and redundant interactions demonstrated by the various molecular cascades involved. Proteomic substrate profiling (degradomics) offers a global strategy to investigate such complex systems [45].

In this study, we demonstrate that, in a time- and space-dependent manner, the ultimate downstream proteolytic activity in plasma from tPA-treated stroke patients differs from that of healthy controls and of patients not receiving tPA. These findings may demonstrate clinical evidence of a thrombolysis-related degradomic signature distinct from that of ischemic progression. Ultimately, this approach may enable us to tease out the components of reperfusion injury versus ischemic injury in progress, or even to discover new targets for ameliorating reperfusion injury. With increasing attempts to extend the treatment window for tPA, and both clinical and imaging characteristics to predict clinical outcome, [11, 12, 14] this approach may add an additional dimension to help us predict responders from non-responders at the bedside.

Proteomic profiling may be especially useful for disease entities such as stroke, since gene expression may not be an accurate reflection of protein expression during cerebral ischemia [34, 46]. Furthermore, targeted study of individual biomarkers, or even panels of biomarkers, requires high patient numbers, poses challenges in generalization to an individual and cannot screen for unknown factors. Other proteomic techniques such as MALDI are faster and simpler but have difficulty identifying the specific proteins involved and may provide non-specific candidates unrelated to the mechanism. In contrast, here we show that a combination of discovery degradomic and targeted proteomic techniques can be robust in a small, well-characterized patient cohort, to offer an overall molecular signature potentially relevant to thrombolysis mechanism.

However, we should emphasize that, since the proteome is intrinsically less stable than the genome, bedside pharmacoproteomic analysis, such as we have attempted in this study, poses its own challenges. To ensure reproducibility and accuracy, we have implemented important factors such as rigorous control–subject matching to just about every clinical characteristic, meticulous sample handling during phlebotomy, rapid freezing to ensure stable samples, and optimization of specific conditions to ensure consistent protease substrate profiling.

Our initial study provides useful proof of concept. But there are several caveats and limitations. First, due to the expense of this type of proteomic profiling, we are limited in the number of patients and samples we can measure, and we, therefore, limited our discovery screening to the important post-tPA time periods that demonstrated the most significant change on SEC. We hope to validate this in a larger number of patients with respect to clinical outcome as we further develop this technology in the future. Second, we measured, by complementary protein microarray, some targeted proteases to ensure sample quality (i.e., no excessive protease degradation) and to confirm the presence of low-abundance proteases known to be implicated in ischemia and reperfusion, such as MMPs and TIMPs. It is by no means a comprehensive list, nor should these measurements be interpreted to have clinical predictive value as in the large studies cited previously with sufficient power. Novel proteins such as BAI-1 and UTRN also need to be validated and quantified in a more in-depth fashion. Third, we chose to study plasma since this is where tPA exerts most of its effect, but plasma is challenging for proteomic profiling due to its complexity over a wide dynamic range, where the high-abundance proteins overwhelm the more interesting low-abundance proteins. We reduced some of the complexity of this mixture by fractionating the plasma prior to proteomic screening. But the gradients of low- versus high-abundance proteins remain a challenge in plasma proteomics. Finally, we had no CSF or brain tissue to offer comparison to the plasma findings, in part, because it is neither feasible nor ethical to obtain these in patients with acute strokes post thrombolysis. But while the target of tPA is within the vessel, in the context of weakened vessels, and injured brain parenchyma, tPA's therapeutic effects may be propagated in the circulation to affect the brain, since we found many proteins relevant to BBB damage. Therefore, it is important to study clinically relevant samples to better understand the plasma landscape by which IV thrombolysis affects clinical outcomes. Future correlations between peripheral and central responses in the CNS may require bedside-back-to-bench translational studies in cell and animal models, using both quantitative and targeted approaches.

In summary, our findings here demonstrate the feasibility of obtaining a composite proteolytic signature of tPA's downstream effects at the bedside using discovery proteomic substrate profiling. These approaches may eventually allow us to gain further understanding of the pleiotropic actions of tPA, and to discover new therapeutic opportunities for increasing the safety and efficacy of reperfusion strategies for stroke therapy. Further in-

depth studies are under way to confirm these findings and to elaborate the comparative plasma profiles with respect to clinical outcome.

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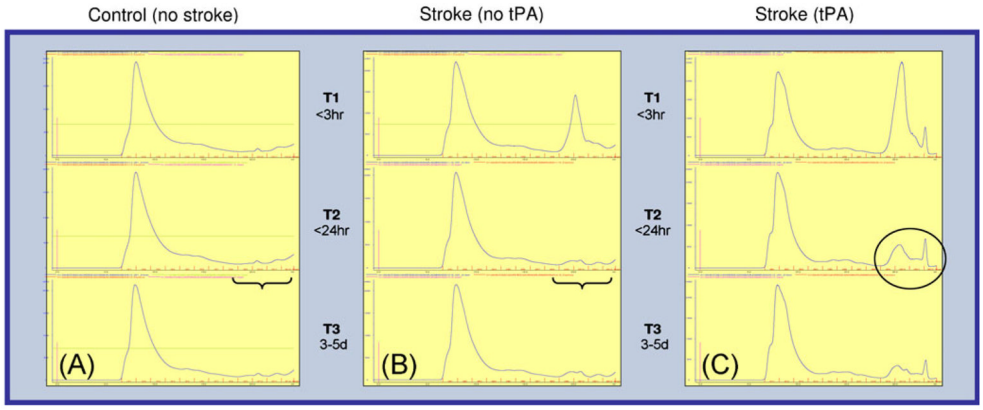

Fig. 1.

UV 214 absorbance chromatogram (degradomic region shown in *brackets*). **a** Control plasma without strokes had a stable substrate profile over time. **b** In acute stroke patients, there are differential expression patterns of smaller proteins as the stroke progresses over time. **c** tPA-treated stroke patients demonstrated different substrate patterns over time from those of untreated patients. Proteins from degradomic fractions of interest (circled) post-tPA are listed in Table 2

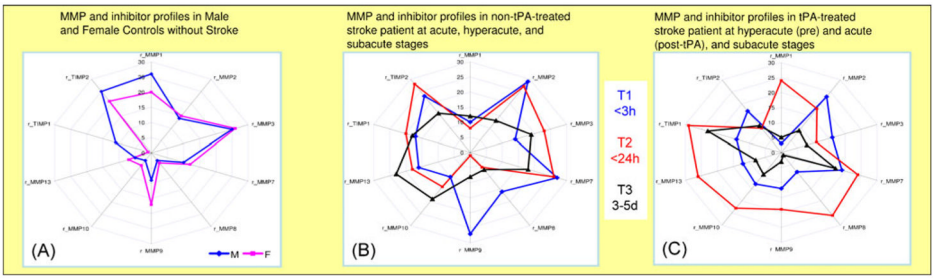

Fig. 2.

Ranked levels of MMP1, 2, 3, 7, 8, 9, 10, and 13 and TIMP1 and 2 using protein antibody microarray in **a** healthy controls (*pink* women; *blue* men) at one time point; **b** stroke patients over time (at three time points from stroke symptom onset); **c** stroke patients treated with tPA over time (at three time points from stroke symptom onset). For **b** and **c** *blue line* hyperacute (*T1* <3 h), *red line* acute (*T2* <24 h), *black lin*e subacute stages (*T3* 3–5 days)

Table 1

Patient clinical characteristics of tPA vs non-tPA vs healthy controls

Clinical characteristics vs patient grouping	tPA stroke patient (*n*=7)	Non-tPA stroke patient (*n*=6)	Healthy control (*n*=4)
Age	66	67	68
Gender (% women)	43%	50%	50%
NIHSS	16	15	0
Time to tPA	2.1 h	N/A	N/A
Hypertension	28%	30%	50%
Diabetes type II	14%	16%	25%
Smoking	0	0	0

Table 2

Partial list of degraded protein fragments found in degradomic fractions post-tPA administration

GI#	NP#	Name
4502005	NP_001613.1	Alpha-2-HS-glycoprotein
4502027	NP_000468.1	Albumin precursor
4502157	NP_001636.1	Apolipoprotein C-I precursor
4502355	NP_001693.1	Brain-specific angiogenesis inhibitor 1 precursor
4503195	NP_000093.1	Cytochrome P450, family 17
4504035	NP_003866.1	Guanine monophosphate synthetase
4504349	NP_000509.1	Beta globin
4504489	NP_000403.1	Histidine-rich glycoprotein precursor
4504781	NP_002206.1	Inter-alpha (globulin) inhibitor H1
4505199	NP_000892.1	Nuclear receptor subfamily 3, group C, member 2
4505375	NP_002490.1	Neogenin homolog 1
4505529	NP_000599.1	Orosomucoid 2
4507249	NP_003464.1	Signal transducing adaptor molecule 1
4507725	NP_000362.1	Transthyretin
4557385	NP_000055.1	Complement component 3 precursor
4557577	NP_001434.1	Fatty acid binding protein 1, liver
4557871	NP_001054.1	Transferrin
4826762	NP_005134.1	Haptoglobin
4885075	NP_005163.1	Atonal homolog 1
5729826	NP_006675.1	Complement factor H-related 4
6005938	NP_009055.1	Utrophin
8922436	NP_060570.1	EF-hand domain (C-terminal) containing 1
9910394	NP_064517.1	1-acylglycerol-3-phosphate *O*-acyltransferase 3
11761629	NP_068657.1	Fibrinogen, alpha chain isoform alpha preproprotein
11967987	NP_071897.1	Fibrosin 1
12007650	NP_072091.1	*N*-deacetylase/*N*-sulfotransferase (heparan glucosaminyl) 4
13376840	NP_079510.1	WD repeat domain 61
14670369	NP_127501.1	Poly(rC) binding protein 4 isoform c
16950609	NP_055899.1	Mitochondrial ribosomal protein S27
21361918	NP_071751.2	Leucine proline-enriched proteoglycan (leprecan) 1
28827805	NP_789786.1	Hypothetical protein LOC202243
31982906	NP_116255.2	Cingulin-like 1
32483410	NP_000574.2	Vitamin D-binding protein precursor
32698982	NP_872386.1	Cytochrome b-561 domain containing 1
34013528	NP_065764.1	Hypothetical protein LOC57473
40354216	NP_787071.2	Sine oculis homeobox homolog 5
42544179	NP_036259.2	Cip1-interacting zinc finger protein
45580723	NP_066275.3	Haptoglobin-related protein
47717131	NP_434700.2	Caspase recruitment domain protein 9

GI#	NP#	Name
48762942	NP_003950.1	Huntingtin interacting protein-1-related
50363221	NP_001002235.1	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antipro)
50659080	NP_001076.2	Serpin peptidase inhibitor, clade A, member 3 precursor
51458810	XP_496420.1	Predicted: similar to heat shock protein HSP 90-alpha (HSP 86)
51472914	XP_370973.2	Predicted: similar to KIAA1501 protein
51476111	XP_496536.1	Predicted: similar to Apolipoprotein A-I precursor (Apo-AI)
52353945	NP_001004750.1	Olfactory receptor, family 51, subfamily B, member 6
62412856	NP_056040.1	BNIP2 motif containing molecule at the carboxyl terminal region 1
66793368	NP_001018857.1	Similar to Zinc finger protein 264
66932947	NP_000005.2	Alpha-2-macroglobulin precursor
66932990	NP_001018088.1	Folylpolyglutamate synthase isoform b
67190748	NP_009224.2	Complement component 4A preproprotein
67782321	NP_001020029.1	Spectrin beta isoform a
70906437	NP_000500.2	Fibrinogen, gamma chain isoform gamma-A precursor